Eur päisches Patentamt
Eur pean Patent Office
Office eur péen des brevets

Bescheinigung Certificate Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

99310188.0

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
THE HAGUE, 07/11/00
LA HAYE, LE

Eur päisches Patentamt | European Patent Office | Office eur péen des brevets

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 99310188.0
Demande n°:

Anmeldetag:
Date of filing: 17/12/99
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
UNILEVER PLC
London EC4P 4BQ
UNITED KINGDOM

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Production of camelid antibodies in plants

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C12N15/82, A01H5/00, C07K1/00

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:
See for original title page 1 of the description.

PRODUCTION OF ANTIBODIES

FIELD OF THE INVENTION

The present invention is in the field of applied biotechnology and relates in particular to an economic way of producing antibodies, or more particularly fragments thereof, in plants.

BACKGROUND OF THE INVENTION

The production of antibodies in microbial or plant systems can be advantageous for a number of applications.

Firstly microbial or plant sources can be used as bioreactors. In this situation the antibodies and/or their derivatives are produced on a large scale in modified organisms. The use of such 'bioreactors', especially plants has the advantage that scale up to produce large quantities e.g. more than 100 kg or even more than 1000 kg of antibodies is relatively easy and does not require significant investments in harvesting or processing equipment.

Alternatively antibodies and/or their derivatives can be produced in plants with the aim of reprogramming the plant metabolism or to improve defence mechanisms of said plant. The antibodies to be produced are chosen such that they target specific enzymes to modulate their activity and/or to provide the plant with protection against pathogens such as parasites and viruses.

Several attempts have been made to express antibodies or fragments thereof in micro-organisms or plants.

Evidence from the literature suggests that where it is desired to express antibodies in microbial systems, the most

favourable results are obtained using smaller fragments. Whole antibodies and larger antibody fragments, such as Fab fragments, are very difficult if not impossible to produce. Smaller molecules such as single chain antibody fragments (scFv) are somewhat easier. Generally, however the best results are obtained if even smaller molecules such as heavy chain variable fragments (HCV) are produced (see, for example, WO 94/25591 (Unilever)).

In plant host systems, by contrast, it appears that although both large molecules, such as complete murine antibodies and smaller murine antibody fragments such as single chain Fv (scFv) fragments are capable of being expressed, in order to achieve stable high level expression with retention of an acceptable degree of binding activity and no detrimental effect on plant cell morphology, then larger molecules, such as complete antibodies or Fab fragments, are preferred.

The production of functional complete murine antibodies in plants was first reported by Hiatt et al, Nature, 342, 76-78 (1989). Subsequent reports of the functional expression of murine monoclonal antibodies in plants include Düring et al, Plant Molecular Biology, 15, 281-293, (1990) and Ma et al, European Journal of Immunology, 24, 131-138, (1994).

Transgenic tobacco plants expressing a synthetic gene encoding an antigen binding single chain Fv protein (scFv) and which produce functional scFv protein have been described by Owen et al, Biotechnology, 10, 790-794, (1992). The expression of functional (that is having antigen-binding activity) scFv protein in transgenic plants has also been described in other reports in the literature, see, for example, Tavlaoraki et al, Nature, 366, 469-472 (1993).

Given that the production of functional complete antibodies requires the correct assembly, via covalent and non-covalent interactions, of both the antibody heavy and light chains, it might have been expected that expression of smaller antibody fragments, with their less stringent assembly requirements, would be advantageous. It has however been reported that generally, in practice, better yields are achieved with plants transformed with complete murine antibodies rather than small fragments (Ma et al, Science, 268, 716-719 (1995))

Furthermore, it is the experience of the present inventors that the expression of genes encoding scFv proteins in plants is not reliably reproducible and hence such a process would not readily lend itself to large scale production. Moreover, the inventors have found that expression of genes encoding scFv molecules in plants can have an undesirable effect on plant cell morphology.

Benevenuto et al, Plant Molecular Biology, 17, 865-874 (1991) describe attempts to express smaller isolated murine heavy chain variable domain antibody fragments in plants. Successful expression is reported but there is no indication that binding affinity is retained.

WO 94/4678 (Casterman et al) describes the isolation of immunoglobulins from Camelids. These immunoglobulins, hereinafter "heavy chain immunoglobulins" have a characteristic distinct structure in that they are naturally devoid of light chains. This in turn leads to a characteristic structure for the heavy chain fragments of these immunoglobulins, because the variable domain has no interaction sites with a light chain fragment and the antigen-binding site is located exclusively in the heavy chain variable domain. The heavy chain variable fragments (HCV) of these immunoglobulins are often referred to as VHH fragments.

Such immunoglobulins or fragments thereof, show the functional properties of conventional, four chain, immunoglobulins but by virtue of their simplified structure offer advantages in preparation and use.

There are no reports in the literature of the expression in plants of heavy chain immunoglobulins or fragments thereof.

There remains a continuing need for the development of improved methods for the production of antibodies, more particularly fragments thereof, with the aim of providing methods suitable for economical large scale production.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect the invention provides a method for modifying a plant to produce antibodies or active fragments or derivatives thereof comprising introducing into a plant one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof, or one or more sequences encoding a protein functionally equivalent thereto.

Also provided is a method for preparing a heavy chain immunoglobulin or an active fragment or derivative thereof comprising extracting said immunoglobulin or fragment or derivative thereof from a plant modified according to the first aspect of the invention.

In an alternative aspect, the invention provides the use of one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof, or one or more sequences encoding a protein functionally equivalent thereto, to modify a plant.

The invention further provides a plant having one or more transgenes encoding one or more heavy chain immunoglobulins or active fragments or derivatives thereof, or one or more sequences encoding a protein functionally equivalent thereto, incorporated, preferably stably, into its genome, and uses thereof.

Also provided is a modified plant having enhanced levels of heavy chain immunoglobulins or active fragments or derivatives thereof, or proteins functionally equivalent thereto, particularly compared to equivalent but unmodified plants.

Seeds, fruits and progeny of such plants and hybrids are included within the invention.

The invention further provides a construct comprising one or more DNA sequences encoding a heavy chain immunoglobulin, or an active fragment or derivative thereof, or one or more sequences encoding a protein functionally equivalent thereto, operably linked to a suitable promoter. The invention also provides plants comprising such a construct together with seeds, fruits and progeny thereof.

Food products such as sauces, dressings, tomato products such ketchups, meals, juices and soups, comprising a plant or part thereof according to the invention are also provided.

Also provided are skin and hair protective compositions and pharmaceutical compositions comprising a plant or part thereof according to the invention.

As used herein, "plant" means a whole plant or part thereof, or a plant cell or group of plant cells, or an extract thereof. The invention is particularly directed at

transforming whole plants and the use of the whole plant or significant parts thereof.

The term "antibody" refers to an immunoglobulin which may be derived from natural sources or synthetically produced, in whole or in part. An "antibody fragment", alternatively an "active fragment", is a portion of a complete antibody or immunoglobulin which retains the ability to exhibit at least part and preferably all of the antigen binding activity.

A "heavy chain immunoglobulin" is an immunoglobulin naturally devoid of any variable light chain domains but which is immunologically active and thus capable of specifically combining with an antigen. The antigen-binding capacity and specificity is located exclusively in the immunoglobulin heavy chains, more specifically in the heavy chain variable domains. The "sequence encoding the heavy chain immunoglobulin or an active fragment or derivative thereof" encompasses a genomic or cDNA clone or a sequence which in proper reading frame encodes an amino acid sequence which is functionally equivalent to the amino acid sequence of the protein encoded by the genomic or cDNA clone. By "functionally equivalent" is meant any protein or fragment or derivative thereof sequence which has the same or similar antigen-binding properties, said antigen-binding capacity being located in a single binding domain. Similarity in functionality can be evaluated by routine screening assays, for example, by assaying the binding affinity of the immunoglobulins produced upon expression in plants.

A "functionally equivalent" derivative may be characterised by an insertion, deletion or substitution of one or more amino acid residues in the sequence of the heavy chain immunoglobulin or active fragment thereof and includes within its scope fusion molecules. Such derivatives may readily be

made by using conventional techniques well known in the art such as peptide synthesis techniques or recombinant DNA manipulation of a gene encoding a heavy chain immunoglobulin by site directed mutagenesis.

It will be appreciated that immunoglobulins or fragments or derivatives thereof modified to enable them to function as binding domains in the same way as immunoglobulins naturally devoid of light chain domains ("heavy chain immunoglobulins") may also suitably be used according to the invention.

A "gene" is a DNA sequence encoding a protein, including modified or synthetic DNA sequences or naturally occurring sequences encoding a protein, and excluding the 5' sequence which drives the initiation of transcription.

"Operably linked to one or more promoters" means the gene, or DNA sequence, is positioned or connected to the promoter in such a way to ensure its functioning. The promoter is any sequence sufficient to allow the DNA to be transcribed. After the gene and promoter sequences are joined, upon activation of the promoter, the gene will be expressed.

An "equivalent, unmodified" plant is a plant which has a substantially identical genotype to a modified plant of the invention excepting the introduced sequences present in the plant of the invention.

A "construct" is a polynucleotide comprising nucleic acid sequences not normally associated in nature.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention may be more fully understood by reference to the following description, when read together with the accompanying drawings in which:

Figure 1 shows the nucleotide sequence (SEQ. ID. No. 1) and corresponding protein sequence (SEQ. ID. No. 2) of a fragment encoding the heavy chain variable domain of an anti-RR6 antibody (denoted HCV33) from a llama with an attached peptide linker group (denoted myc) (RR6 is an azo dye, available from ICI; myc is a peptide comprising the sequence Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn, SEQ. ID. NO. 3).

Figure 2 shows a specificity ELISA assay (A) and SDS-PAGE analysis (B) of whole cell extract of a tobacco plant transformed (Example 4) with the plant expression vector pPV.8 - No leader - HCV33-myc of Example 1. The positive control was at a concentration of 250ng/ml. For the ELISA assay, 50µl of plant extract was used. Total protein concentration in the plant extract was 2ng/ml.

Figure 3 shows a specificity ELISA assay (A) and SDS-PAGE analysis (B) of whole cell extracts of a tobacco plant transformed (Example 5) with the expression vector pPV.8-GBSS-HCV33-myc of Example 1. The positive control was at a concentration of 20µg/ml. Total protein concentration in the plant extract was 1-2mg/ml.

Figure 4 shows the results of immuno-histology studies as reported in Example 5 on a tobacco plant transformed with the expression vector pPV.8-GBSS-HCV33-myc (from Example 1) confirming that HCV33 material is localised in the chloroplasts.

Figure 5 Shows the amount of active antibody present in fruit columella extracts of a tomato plant transformed (Example 5) with the plant expression vector pPV.8-GBSS-HCV33-myc (Example 1) expressed as % of total soluble protein as determined via direct binding ELISA.

Figure 6 shows the nucleotide sequence (SEQ. ID. No. 4) and corresponding protein sequence (SEQ. ID. No. 5) of a fragment encoding the heavy chain variable domain of an anti-potato SBEII antibody (denoted Clone1) with attached peptide linker groups denoted His6 (His-His-His-His-His-His) and myc (as described in Figure 1). (Potato SBEII is a starch branching enzyme also denoted SBE A; Jobling *et al.*, Plant Journal, 18, 163-171 1999).

Figure 7 Shows the amount of active antibody present in whole leaf extract of a tobacco plant transformed with the plant expression vector pSJ35-CERV-GBSS-Clone1-His6-myc of example 1, expressed as % of total soluble protein as determined via direct binding ELISA.

Figure 8 shows the results of immuno-histology studies as reported in Example 6 on a tobacco plant transformed with the expression vector pSJ.34-2x35S-GBSS-scFv3299-hydrophilII and that plastid formation/function is disrupted.

Figure 9 shows a specificity ELISA assay (A) and SDS-PAGE analysis (B) of whole cell extracts of tobacco plants transformed (Example 8) with two further plant expression vectors from Example 1, namely pPV.8-PRIa-HCV33-myc-KDEL (1-6) and pPV.8-GBSS-

HCV33-myc-KDEL (7-9). Total protein concentration in the plant extract was 1-2mg/ml.

Figure 10 shows the results of immuno-histology studies as reported in Example 8 on a tobacco plant transformed with the expression vector pPV.8-PRIa-HCV33-myc-KDEL (from Example 1) confirming that the HCV33 material, directed to the apoplast using the PRIa leader sequence, is localised in the endoplasmic reticulum.

Figure 11 shows the nucleotide sequence (SEQ. ID. No. 6) and corresponding protein sequence (SEQ. ID. No. 7) of a fragment encoding the heavy chain variable domain of an anti-potato SBEII antibody (denoted Clone46) with attached peptide linker groups denoted His6 and myc (as described in Figure 1).

Figure 12 shows that the SBE (starch branching enzyme) activity of whole leaf extracts of 30 tobacco plants independently transformed (Example 9) with the plant expression vector pSJ.35-CERV-GBSS-Clone46-His6-myc is significantly less than that of plants transformed with pSJ.35-CERV-GBSS-HCV33-myc.

Figure 13 shows the nucleotide sequence (SEQ. ID. No. 8) and corresponding protein sequence (SEQ. ID. No. 9) of a fragment encoding the heavy chain variable domain of an anti-GUS antibody (denoted Clone18) with attached peptide linker groups denoted His6 and myc (as described in Figure 1). (GUS is β-glucuronidase, available from Sigma.)

Figure 14 shows the nucleotide sequence (SEQ. ID. No. 10) and corresponding protein sequence (SEQ. ID. No. 11) of a fragment encoding the heavy chain variable domain

of an anti-GUS antibody (denoted Clone18) with an endoplasmic retention signal (Ser-Glu-Lys-Asp-Glu-Leu) attached peptide linker groups denoted His6 and myc (as described in Figure 1).

Figure 15 shows that the GUS (β-glucuronidase) activity of whole leaf extracts of 30 pCJ.102-transformed tobacco plants independently retransformed (Example 10) with the plant expression vector pSJ.35-CERV-Clone18-KDEL-His6-myc is significantly less than that of control pCJ.102 plants.

Figure 16 shows (A) X-glu staining of pollen from a tobacco plant transformed (Example 11) with pSJ.35-Lat52-HCV33-KDEL of Example 1, and (B) X-glu staining of pollen from a tobacco plant transformed (Example 11) with pSJ.35-Lat52-Clone18-KDEL of Example 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the finding that immunologically active immunoglobulins, or active fragments or derivatives thereof, which are devoid of light chain variable domains, can advantageously be expressed at high level in plants with retention of binding activity and no adverse effects on plant growth or morphology. By means of the invention, antibodies and more particularly active fragments thereof, having specificity for a target antigen may conveniently be prepared by a method which is readily amenable to industrial-scale production.

The method of the invention can be used to bring about expression of heavy chain immunoglobulins with retention of binding activity in all compartments of the plant cell, namely plastid, cytoplasm, apoplast and endoplasmic reticulum, with

the highest levels of expression being achieved in the plastid. This gives the ability to modulate the activity of specific enzymes irrespective of where in the cell the enzyme is found.

The expression of active antibodies or fragments or derivatives thereof in the plastid and/or cytoplasm of the host plant cell represent particular advantages of the method of the invention. By contrast, expression of scFv fragments in plastids generally leads to unstable deformed plastids and expression of such fragments in the cytoplasm does not generally give rise to functional activity.

In order to modify the function of an intracellular protein, the antibody or fragment or derivative thereof must be present and active in those compartments of the host plant cell where the target protein is active. In cases where this is the cytoplasm, the reducing environment inhibits the formation of disulphide links in the antibody and so generally antibodies would not be expected to be active in the cytoplasm.

The present inventors have found that even when the levels of expression of heavy chain immunoglobulin or fragment or derivative thereof in the plant cell are low or undetectable by conventional protein chemical methods such as in the case of expression in the cytoplasm, a measurable effect on enzyme activity may still be achieved. The ability to modulate plant enzyme activity without accumulating antibodies in large amounts may be beneficial in some circumstances, for example, in minimising potential deleterious effects on the growth and health of the plant.

The heavy chain immunoglobulins (or fragments or derivatives thereof) to be expressed in accordance to the invention are derived from immunoglobulins which are naturally devoid of

light chains. Especially preferred are immunoglobulins or fragments or derivatives thereof which are obtainable from Camelids as described, for example, in WO 94/4678 above. Heavy chain variable domains derived from immunoglobulins naturally devoid of light chains are particularly preferred for use in the present invention.

Preferred immunoglobulins for use in the invention are obtainable from *Camelids* especially from *Lamas* (for example *Lama Glama*, *Lama Vicugna* or *Lama Paccos*) or from *Camelus* (for example *Camelus dromedarius* or *Camelus bactrianus*).
Conveniently, a functionally equivalent protein shows at least 50% similarity to the respective amino acid sequence, preferably at least 70% similarity, more preferably at least 80%, most preferably 90-100% similarity to the respective amino acid sequence as determined by techniques well known in the art.

The invention also provides for the expression of complex derivative molecules comprising an immunoglobulin or fragment thereof as described above, associated or otherwise connected to one or more other molecules, for example, an enzyme or a further heavy chain variable domain.

It will be appreciated that the invention extends to any plant which is amenable to transformation. Suitable plants include tobacco, peas, potatoes, spinach, tomato and tea.

The DNA sequences of interest will preferably be operably linked (that is, positioned to ensure the functioning of) to one or more suitable promoters which allow the DNA to be transcribed. Suitable promoters, which may be homologous or heterologous to the gene, useful for expression in plants are well known in art, as described, for example, in Weising et

al, (1988), Ann. Rev. Genetics, 22, 421-477). Promoters for use according to the invention may be inducible, constitutive or tissue-specific or have various combinations of such characteristics. Useful promoters include, but are not limited to constitutive promoters such as carnation etched ring virus (CERV), cauliflower mosaic virus (CaMV) 35S promoter, or more particularly the double enhanced cauliflower mosaic virus promoter, comprising two CaMV 35S promoters in tandem (referred to as a "Double 35S" promoter).

It may be desirable to use a tissue-specific or developmentally regulated promoter instead of a constitutive promoter in certain circumstances. A tissue-specific promoter allows for expression of immunoglobulins in certain tissues. Suitable fruit-specific promoters include the tomato E8 promoter (Deikman et al, (1988), EMBO J, 7, 3315-3320), 2A11 (Van Haaren et al, Plant Mol Biol, 21, 625-640), E4 (Cordes et al, (1989), Plant Cell, 1, 1025-1034) and PG (Bird et al, (1988), Plant Mol. Biol., 11, 651-662,) Nicholass et al, (1995), Plant Molecular Biology, 28, 423-435.

The invention provides in a further aspect an expression cassette comprising as operably linked components in the 5'-3' direction of transcription, a promoter functional in a plant cell, one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof or one or more sequences encoding a protein functionally equivalent thereto and a transcriptional and translational termination regulatory region functional in a plant cell.

The promoter and termination regulatory regions will be functional in the host plant cell and may be heterologous (that is, not naturally occurring in the host plant) or homologous (derived from the plant host species) to the plant

cell and the DNA sequence. Suitable promoters which may be used are described above.

The termination regulatory region may be derived from the 3' region of the gene from which the promoter was obtained or from another gene. Suitable termination regions which may be used are well known in the art and include *Agrobacterium tumefaciens nopaline synthase terminator (Tnos)*, *Agrobacterium tumefaciens mannopine synthase terminator (Tmas)* and the *CaMV 35S terminator (T35S)*. Particularly preferred termination regions for use according to the invention include the tobacco ribulose bisphosphate carboxylase small subunit termination region (TrbcS) or the *Tnos* termination region.

Such DNA constructs may suitably be screened for activity by transformation into a host plant via *Agrobacterium* and screening for immunoglobulin levels.

In order to be able to select for plant cells that have integrated the construct, the expression construct may conveniently be joined to a marker which permits screening or selection, according to methods well known in the art.

Conveniently, the expression cassette according to the invention may be prepared by cloning the individual promoter/gene/ terminator units into a suitable cloning vector. Suitable cloning vectors are well known in the art, including such vectors as pUC (Norrander et al, (1983, Gene 26, 101-106), pEMBL (Dente et al (1983), Nucleic Acids Research, 11, 1645-1699), pBLUESCRIPT (available from Stratagene), pGEM (available from Promega) and pBR322 (Bolivar et al, (1977), Gene, 2, 95-113). Particularly useful cloning vectors are those based on the pUC series. The cloning vector allows the DNA to be amplified or manipulated, for example by

joining sequences. The cloning sites are preferably in the form of a polylinker, that is a sequence containing multiple adjacent restriction sites, so as to allow flexibility in cloning.

Suitably, the nucleotide sequences for the genes may be extracted from the Genbank nucleotide database and searched for restriction enzymes that do not cut. These restriction sites may be added to the genes by conventional methods such as incorporating these sites in PCR primers or by sub-cloning.

Preferably the DNA construct according to the invention is comprised within a vector, most suitably an expression vector adapted for expression in an appropriate host (plant) cell. It will be appreciated that any vector which is capable of producing a recombinant plant comprising the introduced DNA sequence will be sufficient.

Suitable vectors are well known to those skilled in the art and are described in general technical references such as Pouwels et al, Cloning Vectors. A laboratory manual, Elsevier, Amsterdam (1986). Particularly suitable vectors include the Ti plasmid vectors.

Transformation techniques for introducing the DNA constructs according to the invention into host cells are well known in the art and include such methods as micro-injection, using polyethylene glycol, electroporation, or high velocity ballistic penetration. A preferred method for use according to the present invention relies on agrobacterium - mediated transformation.

After transformation of the plant cells or plant, those plant cells or plants into which the desired DNA has been incorporated may be selected by such methods as antibiotic

resistance, herbicide resistance, tolerance to amino-acid analogues or using phenotypic markers.

Various assays, well known in the art, may be used to determine whether the plant cell shows the presence of the desired antibody, or fragment thereof. These include binding assay such as an ELISA or radio-immunoassay.

Plant cells transformed according to the invention may be grown in an appropriate culture or cultivated in soil or some other such suitable medium by methods well known in the art. It will be appreciated that the particular procedures adopted will vary depending on the plant species used, such variations being well within the knowledge of the average skilled practitioner.

These plant cells or plants may then conveniently be harvested and the desired antibody product processed using conventional extraction and purification techniques.

Seeds produced from modified plants containing the sequence coding for the desired antibody product can subsequently be grown to generate a progeny population of modified plants from which the desired product can be isolated.

Under some circumstances, it may be desirable to retain the antibody product with the plant rather than extracting and isolating the product. Where the modified plant or part thereof concerned is edible, for example, an antibody which has an effect on ingestion can conveniently be introduced in the form of a food product. In particular, edible plants or parts thereof, modified to incorporate an antibody or fragment or derivative thereof capable of binding a pre-selected antigen may conveniently be used in a method of passively immunising an animal, preferably a mammal and in particular a

human, against said antigen. Particularly suitable antigens in this case will be pathogenic organisms or derivatives or parts thereof.

In addition to well-established application in the areas of diagnosis, therapy and purification, antibodies and fragments or derivatives thereof according to the present invention may suitably by used in methods to modify the properties of the plant in which they are produced. For example, the expression in a plant of functional anti-viral antibody can be useful in reducing pathogenicity and so provide a method of producing viral resistance. Alternatively, the introduction of antibodies into plants may be used to alter or interfere with plant metabolism by modulating the function of proteins present in the plant such as plant antigens and/or block phytohormones and metabolites.

The Invention will be further illustrated by means of the following examples which are provided by way of illustration only.

Techniques used for the modification and analysis of DNA materials were performed using standard procedures well known in the art, as described, for example, in Sambrook et al, "Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor, Laboratory Press 1989 (hereinafter Sambrook), unless otherwise indicated.

Restriction sites are underlined. HCV denotes heavy chain variable domain.

EXAMPLES

Example 1: Construction of HCV expression vectors

The construction of the HCV plant expression plasmids involved several cloning steps. The anti-RR6 HCV gene sequence was isolated from male llamas immunised with BSA-RR6 (as described in WO 99/23221). The sequence of the DNA fragment used in the following example (HCV33; SEQ. ID. No. 1) is given in Figure 1.

pSJ.30

The plasmid pSJ.30 is a derivative of the binary vector pGPTV-KAN (Becker *et al.*, Plant Molecular Biology 20: 1195-1197, 1992) modified as follows: An approximately 750 bp (Sac I, T4 DNA polymerase blunted - Sal I) fragment of pJIT60 (Guerineau *et al.*, *Plant Mol. Biol.* 18: 815-818, 1992) containing the duplicated cauliflower mosaic virus (CaMV) 35S promoter (denoted 2x35S; Cabb-JI strain, equivalent to nucleotides 7040 to 7376 duplicated upstream of 7040 to 7433, Frank *et al.*, *Cell* 21: 285-294, 1980) was cloned into the Hind III (klenow polymerase repaired) - Sal I sites of pGPTV-KAN to create pSJ.30.

pPV.3

The shuttle vector pPV.3 was constructed by removing the HindIII/EcoRI multiple cloning site from pUC19 and replacing it by a synthetic DNA fragment, destroying the original EcoRI and HindIII sites and introducing a SgfI, HindIII, KpnI, EcoRI and XbaI restriction site. The new insert was constructed by annealing the synthetic oligonucleotides PCR.624 and PCR.625 (Table 1) yielding the insert sequence:

PCR.624 (SEQ. ID. NO. 12)

SgfI HindIII KpnI EcoRI XbaI

5'-AGCTGCGATCGCAAGCTTGGTACCGGGAATTCTCTAGA-3'

3'-CGCTAGCGTTCGAACCATGGCCCTTAAGAGATCTTTAA-5'

PCR.625 (SEQ. ID. No. 13)

pPV.5

The KpnI/EcoRI insert from pSJ.30 containing the 2x35S promoter sequence upstream of the GUS gene, followed by the Nos terminator sequence was inserted into the KpnI/EcoRI opened pPV.3 vector yielding the intermediate vector pPV.5.

pPV.5L

The GUS coding sequence was removed from the intermediate vector pPV.5 as a SalI/SacI fragment and replaced by a synthetic DNA fragment introducing a NcoI, NheI and MunI restriction site, while leaving the original SalI/SacI sites intact. The new insert was constructed by annealing the synthetic oligonucleotides PCR.626 and PCR.627 (Table 1) yielding the insert sequence:

PCR.626 (SEQ. ID. No. 14)

SalI NcoI NheI MunI SacI

5'-TCGACCCATGGCCCGCTAGCCAATTGGAGCT-3'

3'-GGGTACCGGGCGATCGGTTAACC-5'

PCR.627 (SEQ. ID. No. 15)

pPV.5LN

The sequence present immediately 5' of the start codon ATG in this vector (CCACCATGG) was replaced by the plant Kozak sequence TAAACCATGG using PCR. Oligonucleotides PCR.640 and

PCR.641 were used to amplify the 189 bp 3' fragment of the 2x35S promoter and modifying the Kozak sequence via PCR.641. The amplified fragment (SEQ. ID. NO. 18) was digested with EcoRV and NcoI and used to replace the EcoRV/NcoI fragment present in the intermediate vector pPV.5L, yielding the intermediate vector pPV.5LN.

PCR.640/PCR.641 amplification product (SEQ. ID. NO. 18)

PCR.640 (SEQ. ID. NO. 16)

CCACCCACGAGGGAACATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGT
GGTGGGTGCTCCCTTGTAGCACCTTTTTCTTCTGCAAGGTTGGTGCAGAAGTTTCGTTCA

EcoRV
GGATTGATGT**GATATC**TCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCA
CCTAACTACACTATAGAGGTGACTGCATTCCCTACTGCTTGTTAGGGTGATAGGAAGCGT

NcoI
AGACCCTTCCTTTATATAAGGAAGTTCATTTCATTTGGAGAGGACCTCGAGTGTAAA**CCA**
TCTGGGAAGGAAATATATTCCTTCAAGTAAAGTAAACCTCTCCTGGAGCTCACATTTGGT

PCR.641 (SEQ. ID. NO. 17)

TGGGAATTC
ACCCTTAAG

pPV.5-PRIa-HCV-myc

The PstI/EcoRI HCV33-myc fragment from pPIC.HCV33-myc (described in WO 99/23221 was inserted into the NcoI/MunI opened vector pPV.5LN together with a 145 bp NcoI/PstI fragment encoding the PRIa leader sequence allowing the in-frame fusion with the 5' framework-1 sequences of the HC-V fragment.

The NcoI/PstI PRIa leader sequence was constructed by annealing the synthetic oligonucleotides PCR.293 (SEQ. ID. NO. 19), PCR.294 (SEQ. ID. NO. 20), PCR.295 (SEQ. ID. NO. 21), PCR.296 (SEQ. ID. NO. 22), and PCR.297 (SEQ. ID. NO. 23) (Table 1). The product of the annealing reaction was cloned as a HindIII/Pst fragment into pUC.19. After verification of the sequence of the synthetic gene fragment (SEQ. ID. NOS. 24-25) the NcoI/PstI fragment was excised and used in subsequent cloning reactions.

NcoI/PstI fragment encoding PRIA leader sequence

```
NcoI
CATGGGATTTGTTCTCTTTTCACAATTGCCTTCATTTCTTCTTGTCTCTACACTTCTCTT
    CCTAAACAAGAGAAAAGTGTTAACGGAAGTAAAGAAGAACAGAGATGTGAAGAGAA
  M  G  F  V  L  F  S  Q  L  P  S  F  L  L  V  S  T  L  L  L

                                                    PstI
ATTCCTAGTAATATCCCACTCTTGCCGTGCCCAGGTGCAGCTGCA
TAAGGATCATTATAGGGTGAGAACGGCACGGGTCCACGTCG
  F  L  V  I  S  H  S  C  R  A  Q  V  Q  L
```

pPV.5-GBSS-HCV-myc

The PstI/EcoRI HCV33-myc fragment from pPIC.HCV33-myc was inserted into the NcoI/MunI opened vector pPV.5LN together with a 257 bp NcoI/PstI fragment encoding the GBSS leader sequence (SEQ. ID. NOS. 26-27) allowing the in-frame fusion with the 5' framework-1 sequences of the HC-V fragment.

NcoI/PstI fragment encoding GBSS leader sequence

```
NcoI
CATGGCAAGCATCACAGCTTCACACCACTTTGTGTCAAGAAGCCAAACTTCACTAGACACCA
    CGTTCGTAGTGTCGAAGTGTGGTGAAACACAGTTCTTCGGTTTGAAGTGATCTGTGGT
  M  A  S  I  T  A  S  H  H  F  V  S  R  S  Q  T  S  L  D  T
```

```
AATCAACCTTGTCACAGATAGGACTCAGGAACCATACTCTGACTCACAATGGTTTAAGGGCT
TTAGTTGGAACAGTGTCTATCCTGAGTCCTTGGTATGAGACTGAGTGTTACCAAATTCCCGA
K  S  T  L  S  Q  I  G  L  R  N  H  T  L  T  H  N  G  L  R  A

GTTAACAAACTTGATGGGCTCCAATCAAGAACTAATACTAAGGTAACACCCAAGATGGCATC
CAATTGTTTGAACTACCCGAGGTTAGTTCTTGATTATGATTCCATTGTGGGTTCTACCGTAG
 V  N  K  L  D  G  L  Q  S  R  T  N  T  K  V  T  P  K  M  A  S

CAGAACTGAGACCAAGAGACCTGGATGCTCAGCTACCATTGTTTGTGGAAAGGGACAGGTGCA
GTCTTGACTCTGGTTCTCTGGACCTACGAGTCGATGGTAACAAACACCTTTCCCTGTCCACGT
  R  T  E  T  K  R  P  G  C  S  A  T  I  V  C  G  K  G  Q  V  Q
```

PstI
GCTGCA
CG
L Q

pPV.5-No leader-HCV33-myc

The PstI/EcoRI HCV33-myc fragment from pPIC.HCV33-myc was inserted into the NcoI/MunI opened vector pPV.5LN together with a synthetic NcoI/PstI DNA fragment allowing the in-frame fusion with the ATG start codon. The synthetic linker was constructed by annealing the synthetic oligonucleotides PCR.652 and PCR.653 (Table 1) yielding the insert sequence:

PCR.652 (SEQ. ID. NO. 28)

```
     NcoI            PstI
5'-CATGCAGGTGCAGCTGCA-3'
   3'-GTCCACGTCG-5'
```

PCR.653 (SEQ. ID. NO. 29)

pPV5.HCV33-myc-SEKDEL retention signal constructs

The HindIII/BstEII Promoter-leader-HCV33 fragment inserts from pPV5-PRIa-HCV33-myc, pPV5-GBSS-HCV33-myc and pPV5-no leader-HCV33-myc was cloned into HindIII/MunI opened pPV5-PRIa-HCV33-

myc vector together with a myc-SEKDEL encoding BstEII/EcoRI PCR fragment thus introducing the sequence SEKDEL at the C-terminal end of the myc-tag, thus yielding pPV.5-PRIa-HCV33-myc-KDEL, pPV.5-GBSS-HCV33-myc-KDEL and pPV.5-No leader-HCV33-myc-KDEL. The PCR fragment was generated using the synthetic oligonucleotides PCR.300 (SEQ. ID. NO. 30)/PCR.690 (SEQ. ID. NO. 31) (see Table 1) and pPV-PRIa-HCV33-myc as a template.

pSJ.34

The BamHI restriction site of the pGPTV-KAN expression vector containing the kanamycin resistance selectable marker gene (Becker *et al.*, Plant Molecular Biology, 20, 1195-1197, 1992) was destroyed by restricting with BamHI followed by a Klenow reaction.

pPV.8 plant transformation vectors

The HindII/EcoRI inserts from vectors pPV.5-PR1a-HCV33-myc, pPV.5-GBSS-HCV33-myc, pPV.5-No leader-HCV33-myc, pPV.5-PR1a-HCV33-myc-KDEL, pPV.5-GBSS-HCV33-myc-KDEL and pPV.5-No leader-HCV33-myc-KDEL, all containing: --2x35S promoter-5'UT region-leader sequence-antibody fragment gene-Nos terminator sequence--, were inserted into the HindIII/EcoRI vector fragment of pSJ.34, yielding pPV.8-GBSS-HCV33-myc, pPV.8-No leader-HCV33-myc, pPV.8-PR1a-HCV33-myc-KDEL, pPV.8-GBSS-HCV33-myc-KDEL and pPV.8-No leader-HCV33-myc-KDEL respectively.

pPV.5LN-2x35S-GBSS-scFv3299-hydrophilII

The PstI/EcoRI (partial) fragment containing scFv3299-hydrophilII (also known as scFv.KC in patent filing WO 96/27612 and described in more detail in WO 99/27386)was inserted into the NcoI/MunI opened vector pPV.5LN together with a 257 bp NcoI/PstI fragment encoding the GBSS leader sequence (as described for pPV.5-GBSS-HCV-myc) allowing the in-frame fusion with the 5' framework-1 sequences of the HC-V fragment.(By way of explanation, the sequence of scFv3299 is

disclosed in WO96/27612 (wherin it is described as FvKC-II), but with the BstEII-SacI linker fragment replaced with a BstEII-SacI fragment encoding a flexible linker yielding the sequence: TVTVSSGGGGSGGGGSGGGGSDIELT (SEQ. ID. NO. 32). The DNA sequence of the linker is described by Jackson *et al.* (Selection of variants of antibodies and other protein molecules using phage display on the surface of bacteriophage fd. : in Protein engineering: A Practical Approach, eds. Rees, Sternberg and Wetzel, publ. IRL Press, Oxford, 1992)

pSJ.34-2x35S-GBSS-scFv3299-hydrophilII

The HindII/EcoRI 2x35S promoter-GBSS-scFV3299-hydrophilII fragment of pPV.5LN-2x35S-GBSS-scFv3299-hydrophilII was inserted into the HindII/EcoRI vector fragment of pSJ.34.

pSJ.89

A HindIII/SacI fragment containing (1x) 35S promoter-GUS.int (Vaneanneyt *et al.*, Mol. Gen. Genet, 220, 245-250, 1990) was inserted into the HindII/SacI vector fragment of pSJ.34. This plant transformation vector with Kan resistance, contains the 35S promoter driving the GUS.int gene.

pSJ.103

The CERV promoter was isolated by PCR from infected *Dianthu barbatus* leaf material obtained from Dr Rene van der Lugt, Research Institute for Plant Protection (IPO-DLO), Binnehaven 5, P.O. Box 9060, NL-6700 GW, Wageningen, Netherlands, using the primers:

CERV1: ATCCTCAACTTCCAATCAGA (5' primer) (SEQ. ID. NO. 33) and CERV2: TTCTTGAGAGATAGCTTGA (3' primer, 14 nt downstream of the transcription start site) (SEQ. ID. NO. 34).

The approx 380 bp fragment (corresponding to nt 6737-7118 of Hull *et al.*, EMBO Journal, 5, 3083-3090, 1986) and was cloned into the TA cloning vector pT7Blue (Invitrogen) with the promoter in the same orientation as the T7 promoter. Sequence analysis showed that the isolated CERV promoter differed from the published sequence at several positions (T-C at 6790, C-T at 6826, A-G at 6872 and T-A at 6729) the most significant of which was a small deletion in a polyA tract in the 5' untranslated leader (4A's vs. 9A's in the published sequence). These changes probably represent differences in virus isolates rather than PCR errors but these cannot be ruled out.

A HindIII - BamHI fragment from pSJ.103 was used as the source of the promoter in plant transformation vectors. Since the HindIII site was 13 nucleotides downstream of the CERV1 oligonucleotide, this sequence was not present in the promoter.

pSJ.108

The HindIII/BamHI CERV promoter fragment of pSJ.103 was inserted into the HindIII/BamHI vector fragment of pSJ.89. This plant transformation vector with Kan resistance, contains the CERV promoter driving the GUS.int gene

pPV.7

The HindIII/EcoRI insert from pSJ.108 containing the CERV promoter sequence upstream of the GUS gene, followed by the Nos terminator sequence was inserted into the HindIII/EcoRI opened pPV.3 vector yielding the intermediate vector pPV.7.

pPV.7L

The GUS coding sequence was removed from the intermediate vector pPV.7 as a BamHI/SacI fragment and replaced by a synthetic DNA fragment introducing a NcoI, NheI and MunI restriction site, while leaving the original BamHI/SacI sites

intact. The new insert was constructed by annealing the synthetic oligonucleotides PCR.628 and PCR.629 (Table 1) yielding the insert sequence:

PCR.628 (SEQ. ID. NO. 35)

BamHI NcoI NheI MunI SacI
5'-GATCCCATGGCCCGCTAGCCAATTGGAGCT-3'
3'-GGTACCGGGCGATCGGTTAACC-5'

PCR.629 (SEQ. ID. NO. 36)

pPV.7LN

The sequence present immediately 5' of the start codon ATG in this vector (GATCCCATGG) was replaced by the plant Kozak sequence TAAACCATGG by replacing the BamHI/NheI fragment present in pPV.7L with a synthetic fragment constructed by annealing the synthetic oligonucleotides PCR.645 and PCR.646 (Table 1) yielding the intermediate vector pPV.7LN.

PCR.645 (SEQ. ID. NO. 37)

BamHI NcoI
NheI
5'-GATCCACCTCGAGTGTAAACCATGGCCCG-3'
3'-GTGGAGCTCACATTTGGTACCGGGCGATC-5'

PCR.646 (SEQ. ID. NO. 38)

pPV7.LN-GBSS-HCV33-myc

The PstI/MunI HCV33-myc fragment of pPV.8-GBSS-HCV33-myc was inserted into the NcoI/MunI opened vector pPV.7LN together with a 257 bp NcoI/PstI fragment encoding the GBSS leader sequence (as described for pPV.5-GBSS-HCV-myc) allowing the in-frame fusion with the 5' framework-1 sequences of the HC-V fragment.

pPV.7LN-GBSS-Clone1-His6-myc

The Clone1-His6-myc sequence was amplified by PCR using oligonucleotides PCR.288 (SEQ. ID. NO. 39) and JWHCV2 (SEQ. ID. NO. 40) (Table 1) from pHEN.Clone1-His6-myc (Figure 6). The PstI/MunI fragment was inserted into the NcoI/MunI opened vector pPV.7LN together with a 257 bp NcoI/PstI fragment encoding the GBSS leader sequence (as described for pPV.5-GBSS-HCV-myc) allowing the in-frame fusion with the 5′ framework-1 sequences of the HC-V fragment.

pPV.7LN-GBSS-Clone46-His6-myc

The Clone46-His6-myc sequence was amplified by PCR using oligonucleotides PCR.288 and JWHCV2 (Table 1) from pHEN.Clone46-His6-myc (Figure 11). The PstI/MunI fragment was inserted into the NcoI/MunI opened vector pPV.7LN together with a 257 bp NcoI/PstI fragment encoding the GBSS leader sequence (as described for pPV.5-GBSS-HCV-myc) allowing the in-frame fusion with the 5′ framework-1 sequences of the HC-V fragment.

pPV.7LN-Clone18-His6-myc

The Clone18-His6-myc sequence was amplified by PCR using oligonucleotides PCR.288 and JWHCV2 (Table 1) from pHEN.Clone18-His6-myc (Figure 11). The NcoI/MunI fragment was inserted into the NcoI/MunI opened vector pPV.7LN allowing the in-frame fusion with the 5′ framework-1 sequences of the HC-V fragment.

pPV.7LN-Clone18-His6-myc-KDEL

The Clone18-His6-myc sequence was amplified by PCR using oligonucleotides PCR.288 and JWHCV1 (SEQ. ID. NO. 41) (Table 1; to generate 3′ KDEL endoplasmic retention signal) from pHEN.Clone18-His6-myc (Figure 11). The NcoI/MunI fragment was inserted into the NcoI/MunI opened vector pPV.7LN allowing the

in-frame fusion with the 5' framework-1 sequences of the HC-V fragment.

pSJ.35

The BamHI restriction site of the pGPTV-HYG expression vector containing the hygromycin resistance selectable marker gene (Becker *et al.*, Plant Molecular Biology 20, 1195-1197, 1992) was destroyed by restricting with BamHI followed by a Klenow reaction.

pSJ.35-CERV plant transformation vectors

The HindII/EcoRI inserts from vectors pPV7.LN-GBSS-HCV33-myc, pPV.7LN-GBSS-Clone1-His6-myc, pPV.7LN-GBSS-Clone46-His6-myc, pPV.7LN-Clone18-His6-myc and pPV.7LN-Clone18-His6-myc-KDEL all containing: --CERV promoter-5'UT region-(no) leader sequence-antibody fragment gene-Nos terminator sequence--, were inserted into HindII/EcoRI vector fragment of pSJ.35, yielding pSJ.35-CERV-GBSS-HCV33-myc, pSJ.35-CERV-GBSS-Clone1-His6-myc, pSJ.35-CERV-GBSS-Clone46-His6-myc, pSJ.35-CERV-Clone18-His6-myc and pSJ.35-CERV-Clone18-His6-myc-KDEL respectively.

pCJ.102

The 5' end of the GUS gene was amplified from pSJ.34 using primers:

Gus mut: AGTCCCCCATGGTACGTCCTGTAGAAACC (creates NcoI site and mutates second amino acid) (SEQ. ID. NO. 42)
Gus 3': CGTTTTCGTCGGTAATCACCATTCC (SEQ. ID. NO. 43)

and the PCR product was restricted with NcoI and EcoRV. This fragment was inserted together with the HindII/NcoI 2x35S promoter fragment of pPV.5LN and the EcoRV/EcoRI 3'GUS-Nos terminator fragment of pSJ.34 into the HindIII/EcoRI vector fragment of pSJ.34.

pSJ.35-Lat52-Clone18-His6-myc

The NcoI/EcoRI Clone18-His6-myc-Nos fragment of pPV.7LN-Clone18-His6-myc was inserted into the SalI/EcoRI vector fragment of pSJ.35 together with the SalI/NcoI Lat52 promoter fragment of pLAT52-7 (Twell *et al.*, Development, 109, 705-13, 1990).

pSJ.35-Lat52-Clone18-His6-myc-KDEL

The NcoI/EcoRI Clone18-His6-myc-KDEL-Nos fragment of pPV.7LN-Clone18-His6-myc-KDEL was inserted into the SalI/EcoRI vector fragment of pSJ.35 together with the SalI/NcoI Lat52 promoter fragment of pLAT52-7 (Twell *et al.*, Development, 109, 705-13, 1990).

pSJ.35-Lat52-HCV33-myc-KDEL

The PstI/SacI HCV33-myc-KDEL fragment of pPV.8-GBSS-HCV33-myc-KDEL was inserted into the SalI/SacI vector fragment of pSJ.35 with the SalI/PstI Lat52 promoter-5' antibody gene fragment of pSJ.35-Lat52-Clone18-His6-myc.

Example 2: Transformation and cultivation of Tobacco N.

Cells of the agrobacterium strain LBA4404 were transformed with the described plasmids using the freeze thaw method (Plant Molecular Biology Manual PMAN A3/7).

Seeds of Nicotiana Tabacum (var. Petit Havana SR1) were surface sterilised for 10 minutes in a solution of 10% sodium hypochlorate, rinsed 3 times in sterile distilled water and planted in Murashige & Skoog (MS) basal medium+ 3% sucrose +0.9% agar.

After 2 weeks growth seedlings were thinned to 2 per vessel; sterile plantlets were maintained by monthly shoot cuttings onto MS basal media +3% sucrose + 0.9% agar.

Discs were punched from leaves of these plants using a sterile cork borer, then incubated for 10 minutes in a culture of the agrobacterium strain LBA4404 containing the described plasmids which had been grown overnight in Lennox Broth (5g/l NaCl, Yeast Extract 10 g/l, 10 g/l Bacto Tryptone, 15g/l agar). The overnight culture was spun down for 10 minutes at 3000G and the growth media replaced by re-suspending the cells in MS Basal medium +3% sucrose.

Discs were removed from the culture and blotted dry on sterile filter paper before plating face down on a nurse culture plate of tobacco cells. This plate was prepared by adding 2 mls of a cell suspension culture of Nicotiana Bethiama onto a petri dish containing 25 mls of MS Salts, B5 vitamins(1mg/l Nicotinicacid, 1mg/lPyridoxine, 10mg/lThiamine, 100mg/l Inositol) +1mg/l 2-4 D,0.2 mg/lBAP & 0.8%agar. The cells were swirled to cover the agar then covered with a sterile filter paper disc.

Infected leaf discs were incubated at 26°C in a light intensity of 2000 lux for 2 days, then removed from the nurse culture plates to selection media (MS Basal media +3% sucrose ,0.2mg/l IAA, 1mg/lBAP, 0.9%agar +cefotaxime 500mg/l & kanamycin100 mg/l and/or hygromycin B 20mg/l). Plates were then incubated at 3000 lux, 26 C ,16 hours day/8 hours night & transferred every 2 weeks to fresh media (as previously). Shoots that appeared from the cut edges of the discs were removed to MS Basal media +3% sucrose +cefotaxime 500mg/l+kanamycin 100mg/l and/or hygromycin B 20mg/l to root. Leaf samples were taken from rooted shoots for testing as described in Example 3.

Example 3: Extraction and detection of HCV material

Extraction of leaf tissue for analysis of anti-RR6 antibody (denoted HCV 33)

Leaf tissue was homogenised with pestle and mortar (for > 0.5gr samples) or 1.5ml eppendorf reaction tubes (0.1-0.5 gr sample) with fitting plastic pestle in the presence of 5ml extraction buffer (200mM Tris-HCl, pH7.5, 5mM EDTA, 0.1% Tween 80, 1 mM PMSF {PhenylMethylSulfonylFluoride}) per 1gr leaf material. Solid material was removed from the homogenate by centrifugation (10 min 13,000g). The cleared supernatant was stored at 4°C. Total protein concentration was determined using the PIERCE BCA Protein Assay Reagent detection system.

Extraction of tomato fruit tissue for analysis of anti-RR6 antibody (denoted HCV33)

Fruit were dissected into peel, flesh and columella (centre of fruit), frozen and ground with a mortar and pestle in liquid nitrogen. Extracts were prepared as for leaf tissue above.

Extraction of leaf tissue for analysis of anti-potato SBEII antibodies (denoted Clone1 and Clone46)

Leaf tissue was homogenised with Polytron probe in 2ml microcentrifuge tubes in the presence of 4 ml SBE extraction buffer (10mM Tris pH 7.5, 5 mM EDTA, 2.5 mM 1,4-dithiothreitol, 0.1% (w/v) sodium metabisulphite, 5% (v/v) glycerol, 2.5% (w/v) polyvinylpolypyrollidone, 1mM PMSF, 1 tablet Complete (Boehringer Mannheim) per 50ml) per 1gr fresh weight. Solid material was removed from the homogenate by centrifugation (10 min 13,000g). The cleared supernatatant was stored at 4°C. Total protein concetrtaion was determined using the BioRad Protein Assay Reagent detection system.

Extraction of leaf tissue for analysis of anti-GUS antibody (denoted Clone18)

As described for analysis of anti-RR6 antibody, except GUS extraction buffer (50 mM phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 10 mM β-mercaptoethanol or 1 mM DTT).

Detection of RR6 binding activity

1) 96 well ELISA plates (Greiner HC plates) were activated overnight at 37°C with 200 µl/well of a 2µg/mL BSA-RR-6 conjugate solution in 0.05 M sodium carbonate buffer pH9.5.

2) Following a single wash with PBST, the ELISA plates were pre-incubated for 1 hour at 37°C with 200µL blocking buffer per well. *Blocking buffer*: 1% BSA in PBS-T

2) Serial dilutions of test samples (100µL) (together with purified, *P.pastoris* produced HCV33-myc standard) were premixed with equal volumes of blocking buffer and added to the ELISA wells. The antibody fragment was allowed to bind to the antigen for 1-2 hours.

3) Unbound antibody fragment was removed by 4 washes with PBS-T.

4) 100 µL of an 1µg/mL solution of the monoclonal anti-myc antibody Nr 4111 (in blocking buffer) was added to each well. Incubate at 37°C for 1 hour.

5) Unbound antibody was removed by 4 washes with PBS-T.

6) 100 µL of an appropriate dilution of an alkaline phosphatase conjugated anti-mouse antibody (in blocking buffer) was added to each well. Incubate at 37°C for 1 hour.

7) Unbound antibody was removed by 4 washes with PBS-T.

8) Alkaline phosphatase activity was detected by adding 100 µL substrate to each well.

Substrate solution: 1mg/mL pNPP in: 1M diethanolamine/ 1mM $MgCl_2$.

Detection of SBEII binding activity

As for RR6 binding activity, except:

1) 100µl /well of 5µg/ml recombinant potato SBEII (Jobling *et al.*, Plant Journal, 18, 163-171, 1999) in PBS overnight at 4°C.

2) Blocking buffer: 1% BSA in TBS-T; all incubations at room temperature.

3) 4 washes with tap water (also for subsequent wash steps).

4) 100µl /well of 1:2,000 dilution of rabbit anti-llama Ig (in blocking buffer).

9) Optical density readings at 410nm were taken in a microtitre plate reader and values were converted to % total soluble protein by comparison to a standard curve of purified antibody binding antigen.

SDS-PAGE and Western blotting

Crude plant extracts (together with purified, *P.pastoris* produced HCV33-myc standard) were analysed on 12% acrylamide gels using the Bio-Rad mini-Protean II system. Samples (40µl) plus 10µl 4xSDS-PAGE loading buffer were mixed and boiled for 5 min before loading onto the gel. The gel was run at 60V initially (30 min) followed by a 1 hour at 180V. Proteins are transferred from polyacrylamide gels to nitrocellulose by electrophoresis. SDS-PAGE and transfer to nitrocellulose was essentially as described in Maniatis et al. (include reference).

Immunostaining Western blots

1) Western blots were incubated in appropriate dilutions of test antibody (anti-myc at 1µg/ml) in PBS-T for 1-2 hours at room temperature with constant agitation.

2) Unbound antibody was removed by 3x 5 minutes washes with PBS-T.

3) The first antibody was detected using an appropriate dilution of an alkaline phosphatase conjugated anti-mouse antibody in PBS-T for 1-2 hours at room temperature with constant agitation.

4) Unbound antibody was removed by 3x 5 minutes washes with PBS-T.

5) Bound antibody was detected by incubating the filters in substrate solution.
Substrate solution: 60µL NBT stock solution (50mg/ml) 30µL BCIP stock solution (50mg/ml) in 10mL : 1M diethanolamine/1mM $MgCl_2$.

6) The colour reaction by stopped by washing the filters with water.

EM studies

Step 1: Sample preparation:
Leaf samples were fixed using 1% Paraformaldehyde+ 0.05% glutaraldehyde in 0.05M sodium phosphate buffer pH6.8, for 4hrs at 4°C, and then washed in phosphate buffer overnight at 4°C. Samples were embedded by first dehydrating the samples through alcohol, 50, 70, 90% (15mins) and finally absolute alcohol (2x30mins). They were then placed in hydrophilic resin [6 parts LRGold resin +_4 parts GMA (low acid) +0.1% benzoin ethyl ether (UV activator). Resin was changed several times over period of three days. Finally, samples were embedded, using freshly prepared resin, in flat bottomed plastic moulds, allowing the resin to polymerise for 24hrs at room temperature, under nitrogen gas, using UV light (360nm).

Step 2: Staining:
Ultrathin leaf tissue sections were collected on collodion (2% in amyl acetate) coated nickel e.m. grids. Grids were floated on 20µl aliquots of TBS/BSA [20mM Tris/HCL buffer pH7.6 +

0.23M NaCl +1 bovine serum albumin] for 20mins at room temperature, following by incubation with 20µl aliquots of primary antibody (anti-myc or anti-hydrophil II, 1µg/ml) in TBS/BSA +0.05% Tween 20 for 18hrs at room temperature. Grids were washed thoroughly using TBS. The primary antibody was detected by incubating the grids with 20µl aliquots of secondary antibody/gold conjugate diluted in TBS/BSA (Goat a Mouse IgG; 5nm diluted 1:200, 10nm diluted 1:100 20nm diluted 1:50) for 60mins at room temperature. The grids were washed thoroughly, using TBS followed by washes with deionised water and dried. Some grids were silver enhanced, prior to poststaining with 2% aqueous Uranyl Acetate, followed by Lead Citrate. Photographs were taken using a either a Jeol 100CX Mk2 transmission electron microscope at 60,000 magnification (Figure 4) or a Jeol 1220 transmission electron microscope at 40,000 magnification (Figure 10).

Assay of SBE activity

All protein samples were diluted to a concentration of $1\mu g\mu l^{-1}$ using SBE extraction buffer. SBE was assayed using the phosphorylase a stimulation assay (Hawker *et al.*, Arch. Biochem. Biophys., 160, 530-551, 1974). The reaction was carried out at 30°C for 1h on 50µg protein in a final volume of 200µl and contained 100mM 2-(N-morpholino)ethanesulphonic acid (MES), 50mM [U-14C]glucose-1-phosphate (370MBq mole-1, ICN), 1.3U rabbit muscle phophorylase a (Sigma), 50µl plant extract at 1mg/ml protein. Glucose polymers were precipitated and washed as described by Hawker *et al.*, Arch. Biochem. Biophys., 160, 530-551 (1974). The final pellet was dissolved in DMSO and counted with ReadySafe-scintillation cocktail (Beckmann).

Assay of GUS activity

All protein samples were diluted to a concentration of $1\mu g\mu l^{-1}$ using GUS extraction buffer. Ten µl samples were then added

to 190µl of 1 x reaction buffer (50 mM phosphate pH 7.0, 0.1% Triton X-100, 10 mM β-mercaptoethanol or 1 mM DTT, 1 mM p-nitrophenyl β-D-glucuronide) contained in the well of a flat-bottomed microtitre plate. The reaction was left to proceed at 37°C until the wells turned visibly yellow. At this point, 80µl of stop solution (2.5 M 2-amino-2-methyl propanediol) was added and the plate read at 410 nm using a Dynex plate reader. GUS activity was calculated based on positive control wells to which known quantities of GUS were added.

Histochemical staining for GUS activity

Tobacco pollen was stained for GUS activity in microtitre wells for up to 1 hour in 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid according to the method of Jefferson *et al.*, EMBO Journal 6: 3901-7, (1987). The buffer was modified by the use of histochemical staining buffer comprising 100 mM sodium phosphate pH 7.0, 10 mM EDTA, and 0.1% (v/v) Triton X-100 (Stomp, In: *Editorial comments*, Vol. 16, No.5. Cleveland: united State Biochemical, 1990). To minimalise spread of the stain to surrounding pollen grains, 5 mM potassium ferricyanide was added to localise staining (Lojda, Histochemie 22: 347-61, 1970; Mascarenhas and Hamilton, Plant Journal 2: 405-8, 1992).

Example 4: Expression of HCV antigen binding activity in the cytoplasm

Small samples of the leaves of 10 individual Tobacco plants transformed with the expression vector pPV.8-No leader-HCV33-myc were tested for the presence of anti-RR6 binding activity using ELISA. One of the tested samples showed low levels of RR-6 binding activity. Detailed analysis of the anti-RR-6 binding activity present in this clone (Nr 943.37) using SDS-PAGE/Western blot analysis, ELISA and total protein concentration measurement showed that HCV33-myc was expressed

as 0.001-0.002% of total protein present (Figure 2 and Table 2).

Example 5: Expression of HCV antigen binding activity in plastids

Small samples of the leaves of 15 individual Tobacco plants transformed with the expression vector pPV.8-GBSS-HCV33-myc were tested for the presence of anti-RR6 binding activity using ELISA. Seven of the tested samples showed high levels of RR-6 binding activity. Detailed analysis of the anti-RR-6 binding activity present in these clones using SDS-PAGE/Western blot analysis, ELISA and total protein concentration measurement showed that HCV33-myc was expressed as 0.5-1.0% of total protein present (Figure 3). From the Western blot it can be concluded that the construct is processed correctly and that the GBSS leader is removed from the HCV fragment. Localisation of the HCV33 material in the chloroplasts was confirmed via immuno-histology studies (Figure 4).

The fruit of 10 individual tomato plants transformed with the expression vector pPV.8-GBSS-HCV33-myc were picked 15 days post-breaker (red stage), dissected and tested for the presence of anti-RR6 binding activity using ELISA. 5 of tested fruit expressed levels of RR6-binding activity which, by comparison to a standard curve of purified HCV33 binding to RR6, were over 0.1% (one was over 0.2%) of total soluble protein (Figure 5).

Similar high levels of expression were obtained with an HC-V antibody of different specificity. Small samples of the leaves of 30 individual tobacco plants transformed with the expression vector pSJ.35-CERV-GBSS-Clone1-His6-myc were tested for the presence of anti-RR6 binding activity using ELISA. 3

of the tested plants expressed levels of RR6-binding activity over 0.1% total soluble protein (Figure 7).

Example 6: Disruption of plastid by scFv expression

Tobacco plants were transformed with expression vector pSJ34-2x35S-GBSS-scFv3299-hydrophilII. After initial detection of high expression of hCG binding activity, these levels were lost - expression was unstable (not shown). Furthermore, immuno-histology studies (Figure 8) suggested that scFv expression had an adverse affect on plastid structure and function, perhaps due to the aggregation of multiple scFv molecules at the plastid membrane

Example 7: Expression of HCV antigen binding activity in the apoplastic space

Small samples of the leaves of 7 individual Tobacco plants transformed with the expression vector pPV.8-PRIa-HCV33-myc were tested for the presence of anti-RR6 binding activity using ELISA. All tested samples showed low levels of RR-6 binding activity. Detailed analysis of the anti-RR-6 binding activity present in this clone (Nr 943.37) using SDS-PAGE/Western blot analysis, ELISA and total protein concentration measurement showed that HCV33-myc was expressed as 0.001-0.002% of total protein present (Table 2).

Example 8: Expression of HCV33 containing the ER retention signal KDEL

Small samples of the leaves of 22, 23 and 12 individual Tobacco plants transformed with the expression vectors pPV.8-no leader-HCV33-myc-KDEL, pPV.8-PRIa-HCV33-myc-KDEL, pPV.8-GBSS-HCV33-myc-KDEL respectively were tested for the presence of anti-RR6 binding activity using ELISA. None, 16 and 3 of

the tested samples respectively, showed medium to high levels of RR-6 binding activity. Detailed analysis of the anti-RR-6 binding activity present in these clones using SDS-PAGE/Western blot analysis, ELISA and total protein concentration measurement showed that HCV33-myc was expressed as 0, 0.4 and 0.05% of total protein present respectively (Figure 5 and Table 2). Localisation of the HCV33 material, that was directed to the apoplast by using the PRIa leader sequence, in the ER was confirmed using immuno-histology studies (Figure 6).

Example 9: Effect of neutralising HC-V expression on SBE activity in tobacco plants

Samples of the leaves of 30 individual tobacco plants transformed with expression vector pSJ.35-CERV-GBSS-Clone46-His6-myc were assayed for SBE activity. Compared to the effect of the HCV33 anti-RR6 antibody in 30 control plants transformed with expression vector pSJ.35-CERV-GBSS-HCV33-myc, the specific anti-potato SBEII antibody Clone46 significantly reduced the SBE activity in the tobacco plants (Figure 12), indicating that the antibody has neutralised the enzyme activity.

Example 10: Effect of neutralising HC-V expression on GUS activity in tobacco leaves

A tobacco line already transformed with expression vector pCJ.102 and shown to contain GUS activity in leaf tissue was retransformed with the expression vector pSJ35-CERV-Clone18-His6-myc. Samples from 27 individual transformed plants were assayed for GUS activity in comparison to control pCJ.102 tobacco lines that had not been retransformed. A graphical representation of the results from measuring GUS activity in control lines and in lines containing Clone 18 is shown in

Figure 13. A difference between the two sets of data is clearly evident, indicating that the presence of the anti-GUS antibody has decreased GUS activity.

Example 11: Effect of neutralising HC-V expression on GUS activity in tobacco pollen

The tobacco line *lat52-gus*, containing the pollen specific promoter *lat52* driving *gus* in a single copy homozygous state, was obtained as a gift from Dr. David Twell (Botany Department, University of Leicester, UK). This line was retransformed with expression vector pSJ.35-Lat52-Clone18-His6-myc. Control lines were obtained by transforming *lat52-gus* tobacco with pSJ.35-Lat52-HCV33-myc. Pollen from 20 lines of each set of transformed tobacco were tested by histochemical staining for GUS activity. Scanned images showing the staining pattern obtained in a control line and a line containing anti-GUS VHH clone 18 are shown in Figure 16. It can quite clearly be seen that GUS activity has been 'knocked-out' by the presence of anti-GUS antibody. Half the pollen retain GUS activity (shown by blue staining) due to segregation of the transgene at meiosis.

Table 1: Oligonucleotides used in the examples

Code	SEQ. ID. NO.	Nucleotide Sequence
PCR.624	12	AGCTGCGATCGCAAGCTTGGTACCGGGAATTCTCTAGA
PCR.625	13	AATTTCTAGAGAATTCCCGGTACCAAGCTTGCTTGCGATCGC
PCR.626	14	TCGACCCATGGCCCGCTAGCCAATTGGAGCT
PCR.627	15	CCAATTGGCTAGCGGGCCATGGG
PCR.640	16	CCACCCACGAGGGAACATCGTG
PCR.641	17	GAATTCCCATGGTTTACACTCGAGGTCCTCTCCAAATGA
PCR.652	28	CATGCAGGTGCAGCTGCA
PCR.653	29	GCTGCACCTG
PCR.293	19	AGCTCCATGGGATTTGTTCTCTTTTCACAATTGCCTTCAT
PCR.294	20	TTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTA
PCR.295	21	ATATCCCACTCTTGCCGTGCCCAGGTGCAGCTGCA
PCR.296	22	GCTGCACCTGGGCACGGCAAGAGTGGGATATTACTAGGAATAAGAGA A
PCR.300	30	CGCAAGACCCTTCCTCTATATAAG
PCR.690	31	GAGCTCGAATTCTTATTATAGCTCATCTTTCTCTGAATTCAGATCCT CTTCTGAGATGAG
PCR.297	23	GTGTAGAGACAAGAAGAAATGAAGGCAATTGTGAAAAGAGAACAAAT CCCATGG
CERV1	33	ATCCTCAACTTCCAATCAGA
CERV2	34	TTCTTGAGAGATAGCTTGA
PCR.288	39	ATTGCCTACGGCAGCCGCTG
JWHCV1	41	TCCAACCAATTGTTATCATAGCTCATCTTTCTCACTATTCAGATCCT CTTCTGAGATGAG
JWHCV2	40	TCCAACCAATTGTTACTATGCGGCCCCATTCAGATCCTCTTCTGAGA TGAG
Gus mut	42	AGTCCCCCATGGTACGTCCTGTAGAAACC
Gus 3'	43	CGTTTTCGTCGGTAATCACCATTCC

Table 2: Summary of HCV expression levels detected. Maximum observed expression levels are expressed as levels of active material as percentage of total extracted protein

Construct	Compartment	Expression Level
pPV.8-no leader-HCV33-myc	cytosol	0.002%
pPV.8-GBSS-HCV33-myc	chloroplast	1.0%
pPV.8-PRIa-HCV33-myc	apoplast	0.001%
pPV.8-no leader-HCV33-myc-KDEL	cytosol/ER	<0.001%
pPV.8-GBSS-HCV33-myc-KDEL	chloroplast/ER	0.05%
pPV.8-PRIa-HCV33-myc-KDEL	apoplast/ER	0.4%
pPV.8-GBSS-HCV33-myc	tomato fruit columella plastid	0.2%
pSJ.35-CERV-GBSS-Clone1-His6-myc	chloroplast	0.2%

CLAIMS

1. A method for modifying a plant to produce antibodies or active fragments or derivatives thereof comprising introducing into said plant one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof or one or more sequences encoding a protein functionally equivalent thereto, each DNA sequence being operably linked to one or more promoters.

2. A method according to claim 1 wherein the DNA sequence encoding the heavy chain immunoglobulin or fragment or derivative thereof is obtainable from camelids.

3. A method according to claim 1 or claim 2 wherein the plant is selected from tobacco, pea, potato, spinach, tomato or tea.

4. A method according to any one of claims 1 to 3 wherein the heavy chain immunoglobulin or active fragment or derivative thereof binds to a protein present in the plant.

5. A method according to any one of claims 1 to 3 wherein the heavy chain immunoglobulin or active fragment or derivative thereof binds to a plant or animal pathogen.

6. A modified plant having one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof, or one or more sequences encoding a protein functionally equivalent thereto incorporated into its genome.

7. A plant according to claim 6 prepared according to the method of any one of claims 1 to 5.

8. A DNA construct comprising one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof or one or more sequences encoding a protein functionally equivalent thereto, each DNA sequence being operably linked to a promoter.

9. A plant comprising a DNA construct according to claim 8.

10. A modified plant having enhanced levels of heavy chain immunoglobulins or active fragments or derivatives thereof or proteins functionally equivalent thereto compared to equivalent but unmodified plants.

11. Seeds, fruits, progeny and hybrids of a plant according to any one of claims 7,. 9 or 10.

12. A food product comprising a plant according to any one of claims 7, 9, 10 or 11.

13. Use of one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof or one or more sequences encoding a protein functionally equivalent thereto to modify a plant.

14. Use according to claim 13 to increase pathogen resistance in a plant.

15. Use according to claim 13 to modulate plant metabolism.

16. A method for preparing a heavy chain immunoglobulin or an active fragment or derivative thereof comprising the steps of:

(i) modifying a plant according to the method of any of claims 1 to 5, and

(ii) extracting from said modified plant the heavy chain immunoglobulin or active fragment or derivative thereof produced therein.

ABSTRACT

A method for modifying a plant to produce antibodies or active fragments or derivatives thereof comprising introducing into a plant one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof or one or more sequences encoding a protein functionally equivalent thereto, constructs for use in such a method and plants so modified are disclosed.

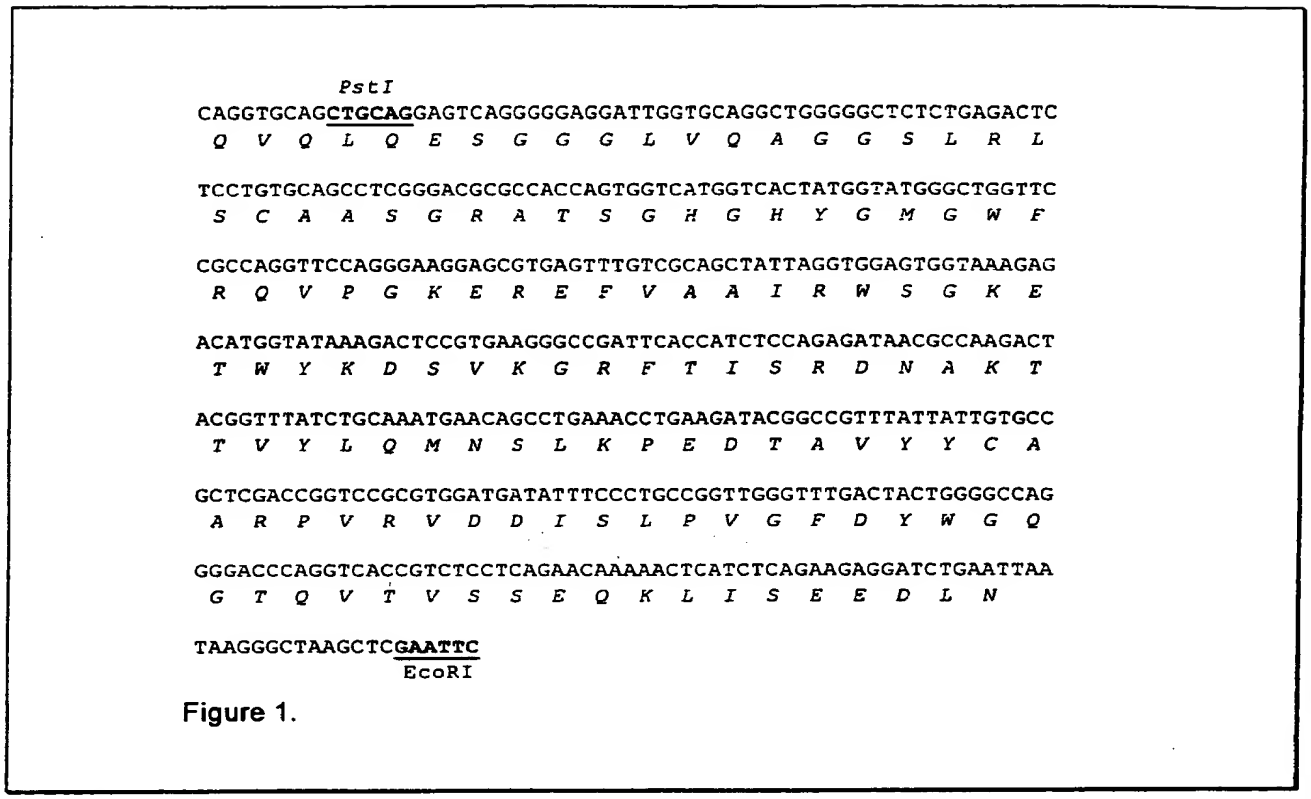

PstI
CAGGTGCAGCTGCAGGAGTCAGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGAGACTC
Q V Q L Q E S G G G L V Q A G G S L R L
TCCTGTGCAGCCTCGGGACGCGCCACCAGTGGTCATGGTCACTATGGTATGGGCTGGTTC
S C A A S G R A T S G H G H Y G M G W F
CGCCAGGTTCCAGGGAAGGAGCGTGAGTTTGTCGCAGCTATTAGGTGGAGTGGTAAAGAG
R Q V P G K E R E F V A A I R W S G K E
ACATGGTATAAAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGATAACGCCAAGACT
T W Y K D S V K G R F T I S R D N A K T
ACGGTTTATCTGCAAATGAACAGCCTGAAACCTGAAGATACGGCCGTTTATTATTGTGCC
T V Y L Q M N S L K P E D T A V Y Y C A
GCTCGACCGGTCCGCGTGGATGATATTTCCCTGCCGGTTGGGTTTGACTACTGGGGCCAG
A R P V R V D D I S L P V G F D Y W G Q
GGGACCCAGGTCACCGTCTCCTCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATTAA
G T Q V T V S S E Q K L I S E E D L N
TAAGGGCTAAGCTCGAATTC
EcoRI

Figure 1.

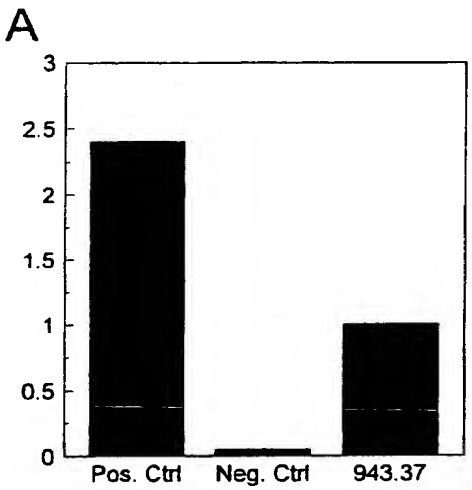
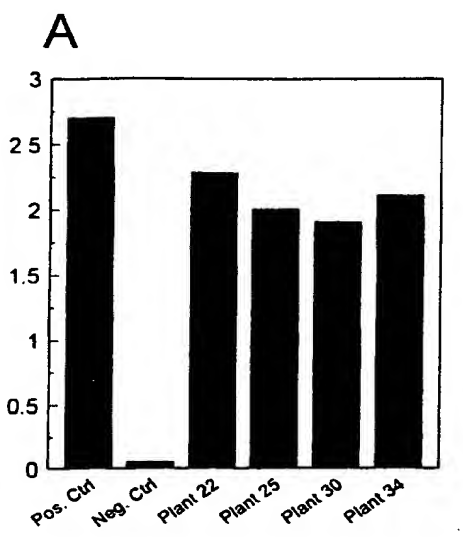
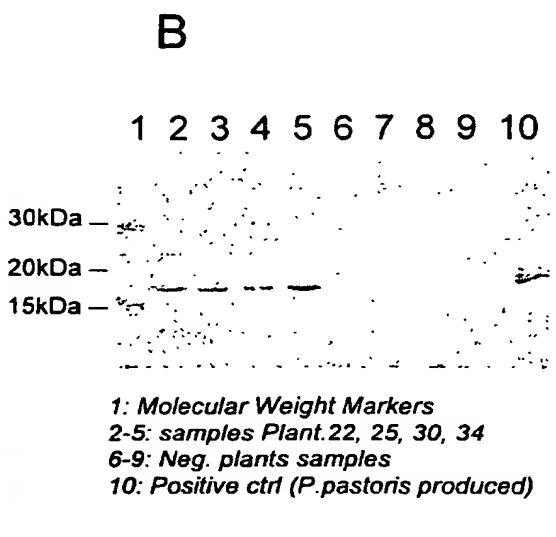

A
3
2.5
2
1.5
1
0.5
0
Pos. Ctrl
Neg. Ctrl
943.37
B
1 2 3
30kDa
20kDa
15kDa
1: Molecular Weight Markers
2: 943.37 sample
3: Neg. Ctrl

Figure 2.





1: Molecular Weight Markers
2-5: samples Plant.22, 25, 30, 34
6-9: Neg. plants samples
10: Positive ctrl (P.pastoris produced)

Figure 3. .

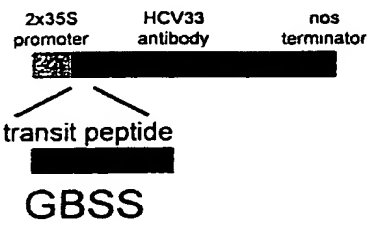

2x35S
promoter
HCV33
antibody
nos
terminator
transit peptide
GBSS

Figure 4.

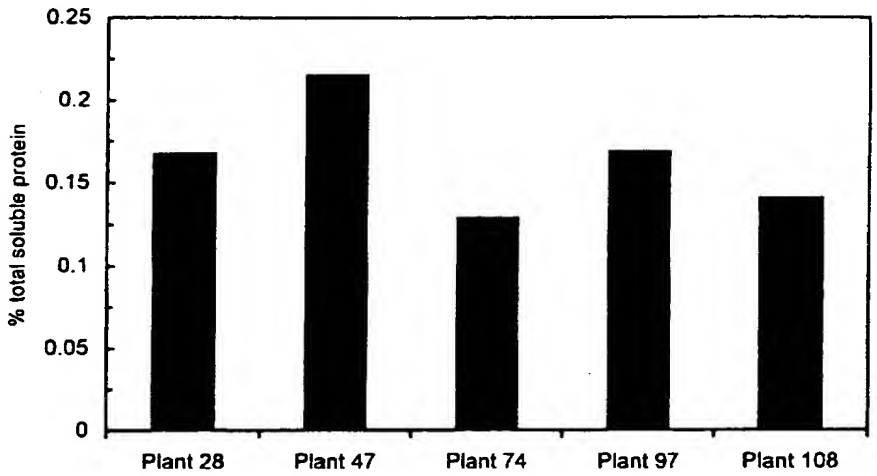

0.25
0.2
0.15
0.1
0.05
0
% total soluble protein
Plant 28
Plant 47
Plant 74
Plant 97
Plant 108

Figure 5.

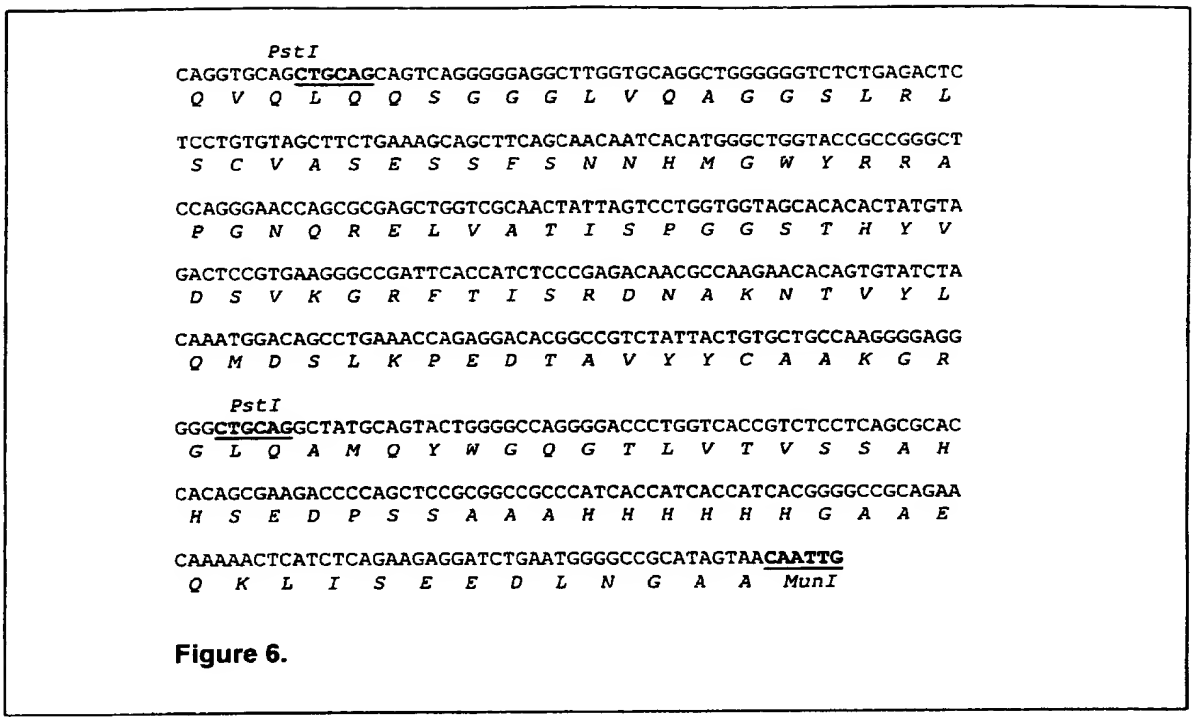

PstI
CAGGTGCAGCTGCAGCAGTCAGGGGGAGGCTTGGTGCAGGCTGGGGGGTCTCTGAGACTC
Q V Q L Q Q S G G G L V Q A G G S L R L
TCCTGTGTAGCTTCTGAAAGCAGCTTCAGCAACAATCACATGGGCTGGTACCGCCGGGCT
S C V A S E S S F S N N H M G W Y R R A
CCAGGGAACCAGCGCGAGCTGGTCGCAACTATTAGTCCTGGTGGTAGCACACACTATGTA
P G N Q R E L V A T I S P G G S T H Y V
GACTCCGTGAAGGGCCGATTCACCATCTCCCGAGACAACGCCAAGAACACAGTGTATCTA
D S V K G R F T I S R D N A K N T V Y L
CAAATGGACAGCCTGAAACCAGAGGACACGGCCGTCTATTACTGTGCTGCCAAGGGGAGG
Q M D S L K P E D T A V Y Y C A A K G R
PstI
GGGCTGCAGGCTATGCAGTACTGGGGCCAGGGGACCCTGGTCACCGTCTCCTCAGCGCAC
G L Q A M Q Y W G Q G T L V T V S S A H
CACAGCGAAGACCCCAGCTCCGCGGCCGCCCATCACCATCACCATCACGGGGCCGCAGAA
H S E D P S S A A A H H H H H H G A A E
CAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAGTAACAATTG
Q K L I S E E D L N G A A MunI

Figure 6.

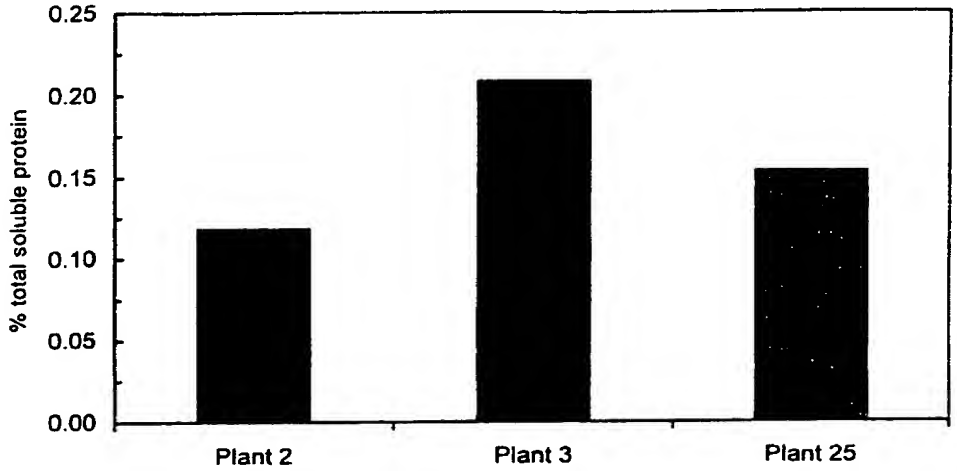

0.25
0.20
0.15
0.10
0.05
0.00
% total soluble protein
Plant 2
Plant 3
Plant 25

Figure 7.

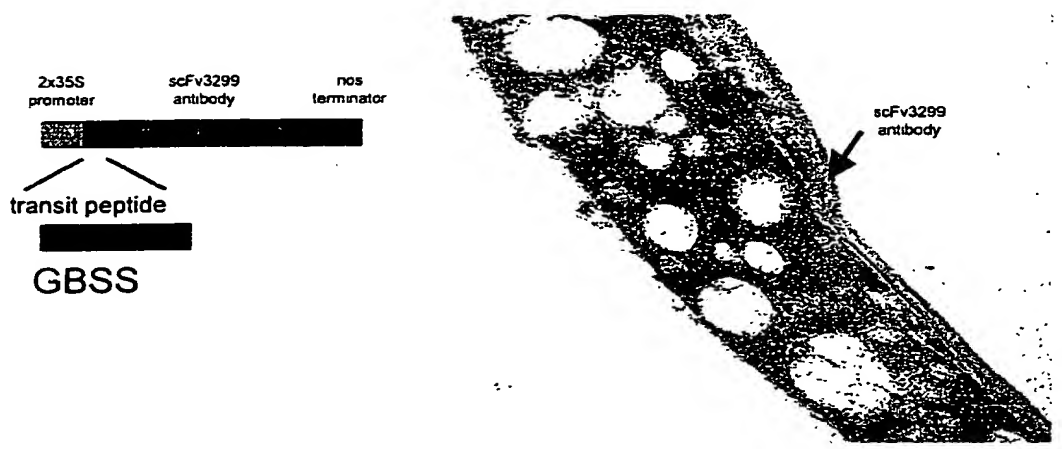

2x35S promoter
scFv3299 antibody
nos terminator
transit peptide
GBSS
scFv3299 antibody

Figure 8.

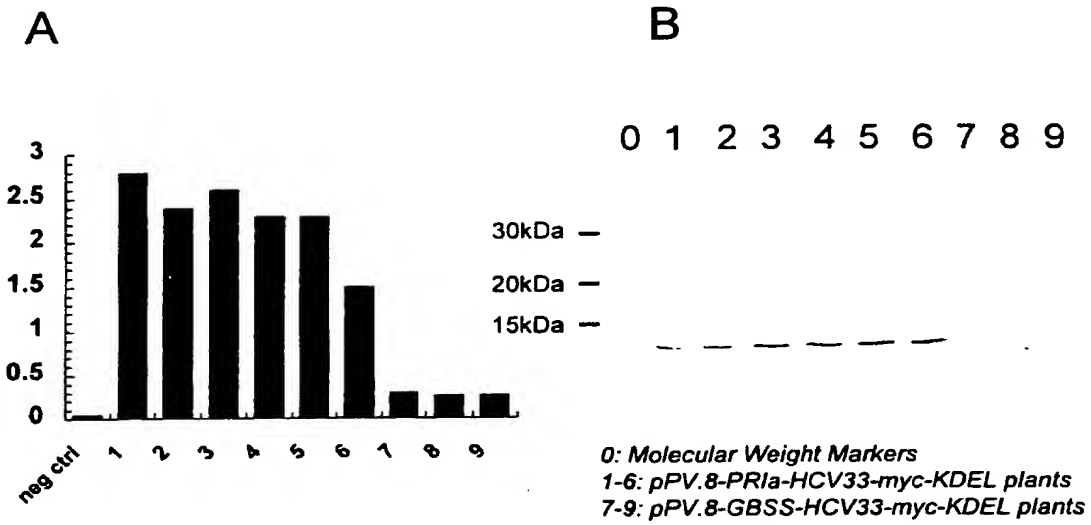

A
B
0 1 2 3 4 5 6 7 8 9
3
2.5
2
1.5
1
0.5
0
30kDa
20kDa
15kDa
neg ctrl 1 2 3 4 5 6 7 8 9
0: Molecular Weight Markers
1-6: pPV.8-PRIa-HCV33-myc-KDEL plants
7-9: pPV.8-GBSS-HCV33-myc-KDEL plants

Figure 9.

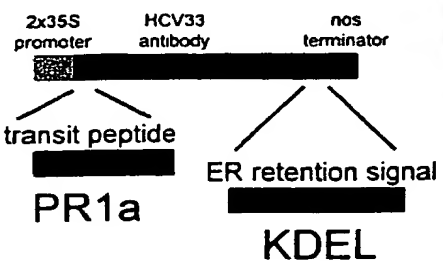

2x35S promoter
HCV33 antibody
nos terminator
transit peptide
PR1a
ER retention signal
KDEL

Figure 10.

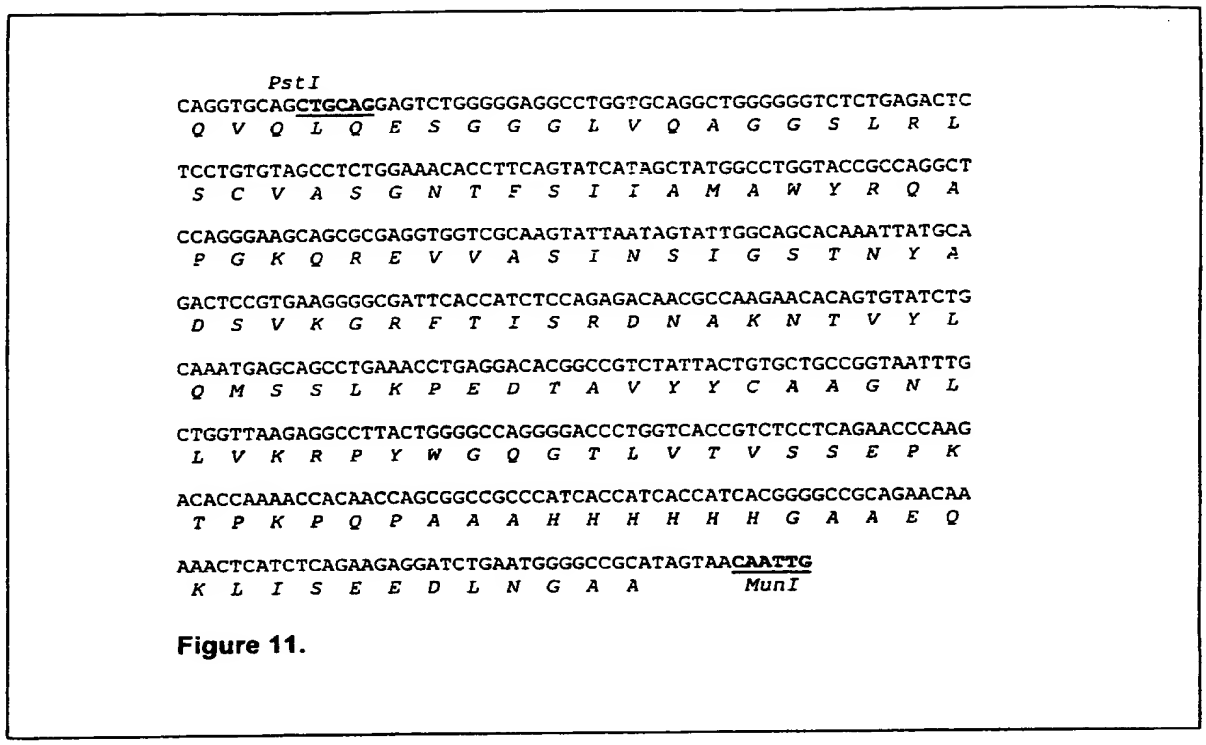

PstI
CAGGTGCAGCTGCAGGAGTCTGGGGGAGGCCTGGTGCAGGCTGGGGGGTCTCTGAGACTC
Q V Q L Q E S G G G L V Q A G G S L R L
TCCTGTGTAGCCTCTGGAAACACCTTCAGTATCATAGCTATGGCCTGGTACCGCCAGGCT
S C V A S G N T F S I I A M A W Y R Q A
CCAGGGAAGCAGCGCGAGGTGGTCGCAAGTATTAATAGTATTGGCAGCACAAATTATGCA
P G K Q R E V V A S I N S I G S T N Y A
GACTCCGTGAAGGGGCGATTCACCATCTCCAGAGACAACGCCAAGAACACAGTGTATCTG
D S V K G R F T I S R D N A K N T V Y L
CAAATGAGCAGCCTGAAACCTGAGGACACGGCCGTCTATTACTGTGCTGCCGGTAATTTG
Q M S S L K P E D T A V Y Y C A A G N L
CTGGTTAAGAGGCCTTACTGGGGCCAGGGGACCCTGGTCACCGTCTCCTCAGAACCCAAG
L V K R P Y W G Q G T L V T V S S E P K
ACACCAAAACCACAACCAGCGGCCGCCCATCACCATCACCATCACGGGGCCGCAGAACAA
T P K P Q P A A A H H H H H H G A A E Q
AAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAGTAACAATTG
K L I S E E D L N G A A
MunI

Figure 11.

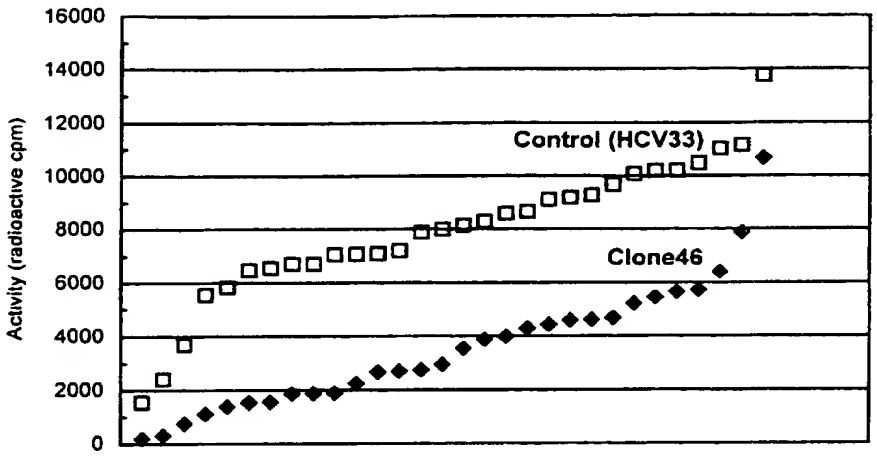

16000
14000
12000
10000
8000
6000
4000
2000
0
Activity (radioactive cpm)
Control (HCV33)
Clone46

Figure 12.

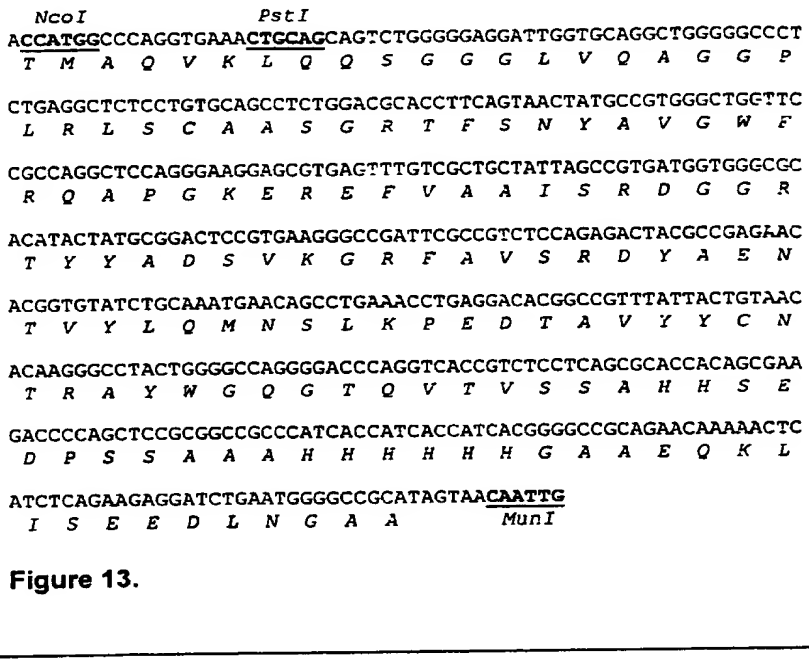

NcoI
PstI
ACCATGGCCCAGGTGAAACTGCAGCAGTCTGGGGGAGGATTGGTGCAGGCTGGGGGCCCT
T M A Q V K L Q Q S G G G L V Q A G G P
CTGAGGCTCTCCTGTGCAGCCTCTGGACGCACCTTCAGTAACTATGCCGTGGGCTGGTTC
L R L S C A A S G R T F S N Y A V G W F
CGCCAGGCTCCAGGGAAGGAGCGTGAGTTTGTCGCTGCTATTAGCCGTGATGGTGGGCGC
R Q A P G K E R E F V A A I S R D G G R
ACATACTATGCGGACTCCGTGAAGGGCCGATTCGCCGTCTCCAGAGACTACGCCGAGAAC
T Y Y A D S V K G R F A V S R D Y A E N
ACGGTGTATCTGCAAATGAACAGCCTGAAACCTGAGGACACGGCCGTTTATTACTGTAAC
T V Y L Q M N S L K P E D T A V Y Y C N
ACAAGGGCCTACTGGGGCCAGGGGACCCAGGTCACCGTCTCCTCAGCGCACCACAGCGAA
T R A Y W G Q G T Q V T V S S A H H S E
GACCCCAGCTCCGCGGCCGCCCATCACCATCACCATCACGGGGCCGCAGAACAAAAACTC
D P S S A A A H H H H H H G A A E Q K L
ATCTCAGAAGAGGATCTGAATGGGGCCGCATAGTAACAATTG
I S E E D L N G A A
MunI

Figure 13.

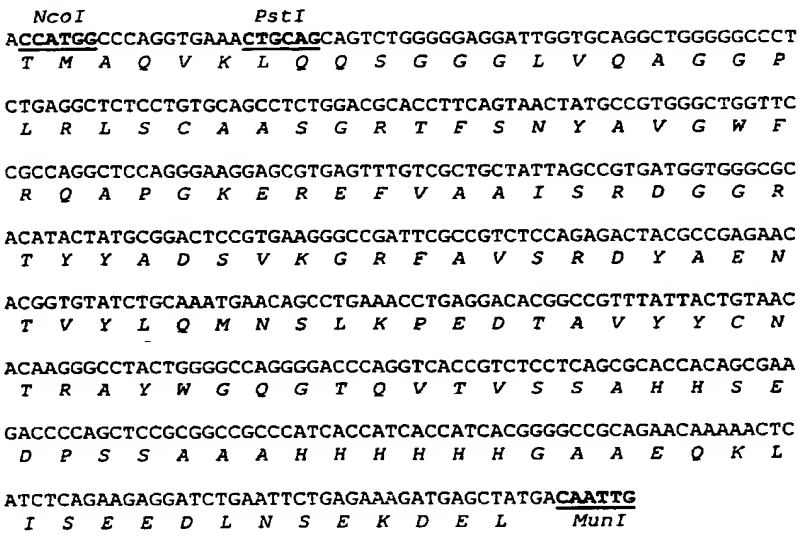

NcoI
PstI
ACCATGGCCCAGGTGAAACTGCAGCAGTCTGGGGGAGGATTGGTGCAGGCTGGGGGCCCT
T M A Q V K L Q Q S G G G L V Q A G G P
CTGAGGCTCTCCTGTGCAGCCTCTGGACGCACCTTCAGTAACTATGCCGTGGGCTGGTTC
L R L S C A A S G R T F S N Y A V G W F
CGCCAGGCTCCAGGGAAGGAGCGTGAGTTTGTCGCTGCTATTAGCCGTGATGGTGGGCGC
R Q A P G K E R E F V A A I S R D G G R
ACATACTATGCGGACTCCGTGAAGGGCCGATTCGCCGTCTCCAGAGACTACGCCGAGAAC
T Y Y A D S V K G R F A V S R D Y A E N
ACGGTGTATCTGCAAATGAACAGCCTGAAACCTGAGGACACGGCCGTTTATTACTGTAAC
T V Y L Q M N S L K P E D T A V Y Y C N
ACAAGGGCCTACTGGGGCCAGGGGACCCAGGTCACCGTCTCCTCAGCGCACCACAGCGAA
T R A Y W G Q G T Q V T V S S A H H S E
GACCCCAGCTCCGCGGCCGCCCATCACCATCACCATCACGGGGCCGCAGAACAAAAACTC
D P S S A A A H H H H H H G A A E Q K L
ATCTCAGAAGAGGATCTGAATTCTGAGAAAGATGAGCTATGACAATTG
I S E E D L N S E K D E L
MunI

Figure 14.

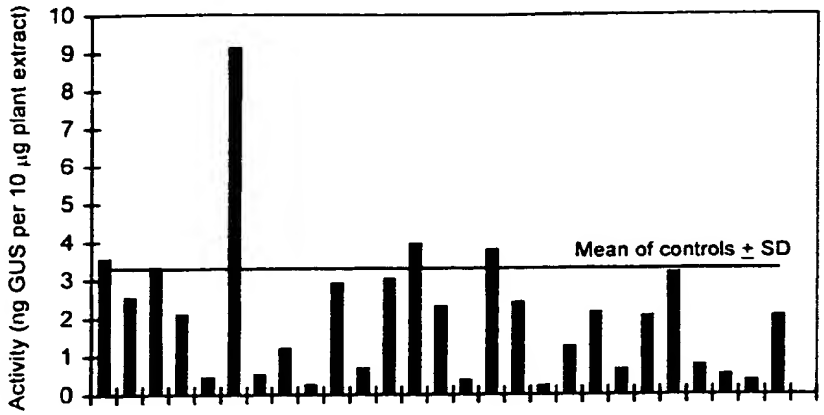

Activity (ng GUS per 10 µg plant extract)
10
9
8
7
6
5
4
3
2
1
0
Mean of controls ± SD

Figure 15.

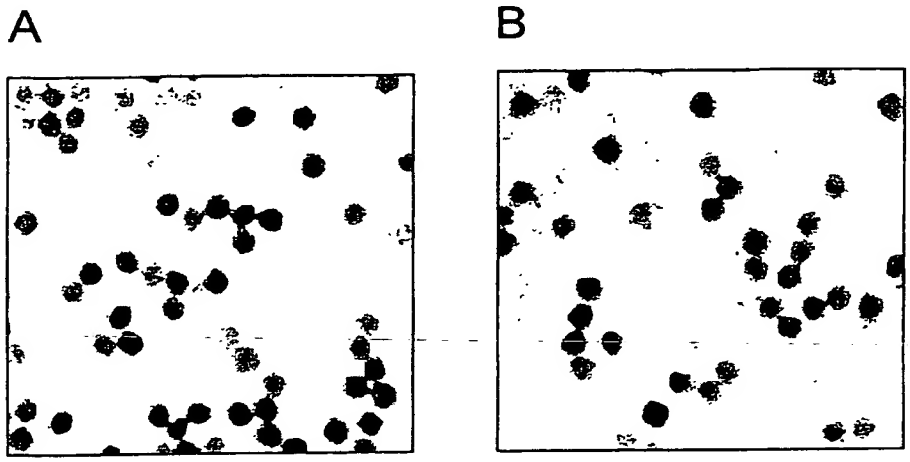


Figure 16.